THE ELIMINATION OF SULFUR FROM COAL BY MICROBIAL ACTION

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INTRODUCTION

The sulfur present in coals in the form of iron pyrites is of interest for at least three reasons: (1) as a contaminant in coals used for the preparation of metallurgical coke, (2) as a contaminant in coal used for power purposes, and (3) as the ultimate source of the sulfur appearing as sulfuric acid in the effluents of mines producing acid waters. Certain bacteria are known which, when grown in the presence of pyrites, copper sulfides, or molybdenum sulfides, catalyze the formation of sulfuric acid with concurrent release of the metallic cation in a soluble form (1-3). Microbiological studies have thus far shown that one of these organisms, Ferrobacillus ferrooxidans, catalyzes the reaction:

$$4FeSO_4 + 2H_2SO_4 + O_2 \longrightarrow 2Fe_2(SO_4)_3 + 2H_2O \quad (1).$$

Experimentally, it has been demonstrated that iron- and/or sulfur-oxidizing organisms catalyze the production of acid and soluble iron from coal sulfur ball material (1) or from the sulfuritic constituents of finely ground coal (2). It is therefore believed that a thorough microbiological study of the oxidation of pyrites would not only clarify the role microorganisms play in the process but conceivably, through strain selection, determination of nutritional requirements, and control of the physical environment, a method for the removal of pyritic sulfur from coal by a microbial process might be devised. Such a study would also clarify the role of microorganisms in the formation of acid mine waters. A related problem, the treatment of acid waters by a microbial process utilizing the unique abilities of the bacterium Desulfovibrio desulfuricans to reduce sulfates to elemental sulfur and/or sulfide, might possibly be carried out by extension of the aforementioned line of attack.

There is very little known concerning the activities and abilities of microbes which may attack organic sulfur-containing compounds other than the naturally-occurring amino acids and vitamins. A study of the oxidation of aromatic sulfur compounds by microorganisms has been initiated in the hope of finding a way of eliminating organic sulfur from coal.

EXPERIMENTAL

A. Materials and Methods

1. Stock Cultures. Stock cultures of the following autotrophic strains of bacteria have been obtained from the Syracuse University Culture Collection. (a) Ferrobacillus ferrooxidans - Culture capable of oxidizing ferrous iron to ferric state. Maintained on medium 9K (see below) containing $FeSO_4$ as energy source. (b) Thiobacillus thiooxidans - Culture capable of oxidizing elemental sulfur to sulfate. Maintained on medium 9KS° and ThS. Strains of the heterotrophic Pseudomonas aeruginosa were those reported previously (4).

2. Media Used. The following media are used for the routine culture of the above bacteria and/or were used in the screening studies with the acid mine water samples:

(a) Medium 9K (5) for iron bacteria: Solution A - Basal Salts - $(NH_4)_2SO_4$, 3.00 g.; KCl, 0.10 g.; K_2HPO_4, .50 g.; $MgSO_4 \cdot 7H_2O$, .50 g.; $Ca(NO_3)_2$, .01 g.; $10NH_2SO_4$, .10 ml.; distilled water, 700 ml. Solution B - Energy Source - $FeSO_4 \cdot 7H_2O$: 300 ml. of a 14.74% (w/v) solution is added to 700 ml. of the basal salts solution to make one liter of medium. The pH is 3.0 to 3.6 without further adjustment.

(b) Medium 9KS 0 - For sulfur oxidizers. As in (a) with 1-2% elemental sulfur (sterilized separately by autoclaving at 100° C. for 3 hours) replacing the iron salt as source of energy; pH adjusted to 3.5.

(c) Medium 9KS - For sulfur oxidizers. As in (a) with 1.0% Na_2S as $Na_2S \cdot 9H_2$) as source of energy.

(d) Medium 9KP - For iron or sulfur oxidizers. As in (a) with 5% pyrite (Sample No. PS-34-6008 obtained in a pulverized form from Bituminous Coal Research, Inc.) as source of energy. This medium was prepared at two pH levels: 3.5 and 7.0.

(e) Medium ThS - For sulfur oxidizers (6).

(f) Medium SS-1 - For sulfate reducers (6).

3. Bacteriological Methods

Standard plate count methods for numbers of heterotrophs were used with nutrient agar (Difco) or Czapek's medium in Petri plates. Tubes of media for determination of autotrophs were examined visually or subjected to microscopic examination. Liquid media for isolation of sulfate-reducing bacteria were prepared in Virtis anaerobic tubes. Agar media were prepared as deep tubes or drawn into foot lengths of 4 mm. OD pyrex tubing. The ends of the latter were sealed with paraffin wax. Growth in these tubes is recognized visually by the production of a black precipitate of iron sulfide.

4. Cultures for Manometric Experiments

Preparations of resting cells of iron and sulfur oxidizers were made according to the methods of Silverman and Lundgren (5,7); heterotrophs were grown as previously noted (4). The Warburg apparatus was used in the conventional manner to measure oxygen uptake (8). Description of the flask contents for any particular experiment is found in the figure legend.

5. Determinations of iron were made by the colorimetric o-phenanthroline method, as described in ASTM methods. A standard curve for concentrations between 20 to 240 γ of iron was prepared, reading optical density at 500 mμ in a Beckman Spectronic 20 spectrophotometer-colorimeter.

6. The source, description, and analyses of the pyritic materials is shown in Table I. The material was used as originally received (passing 65 mesh) or ground in an agate mortar to pass a 325-mesh screen. The crystalline form of the sulfuritic material in these samples was verified as pyrite by X-ray diffraction. In addition, samples of museum-grade pyrite and marcasite ground to -325 mesh were used.

Table I. Analyses of pyritic material

Sample No.	Description and source	Percent pyrite
29	Waste material—pyrite concentrate from coal from Tabo bed, Power Mine, Montrose, Henry County, Missouri.	77.0
30	Waste material—pyrite concentrate from Illinois #6 coal, Peabody #10 Mine, Christian County, Illinois.	60.0
34	Pyrite concretion (sulfur ball) from Pittsburgh bed, Osage #3 Mine, Monongalia County, West Virginia.	54.4[a]
35	Pyrite concretion (sulfur ball) from Meigs Creek #9 coal, Bradford #1 Mine, Harrison County, Ohio.	74.5

a Contained 18.8% $CaCO_3$ in the form of calcite.

B. Proceduresand Results

Samples of acid mine muds were obtained from shafts of the Hutchinson Mine near Irwin, Pa., and waters and bottom muds from the point of exit of the mine waters, and the acid swamp these waters form (Marchand Pool). The pool empties into Sewickley Creek, from which samples were obtained upstream from the pool entry. The samples collected are listed in Table II.

Table II. Samples of acid mine waters and related materials

Sample No.	Source	Description	pH
1	Hutchinson Mine	Acid gob (yellow mud)	2.9
2	Hutchinson Mine	Alkaline gob (orange mud)	6.3
3	Sewickley Creek,	Surface water	3.8
4	upstream from	Bottom mud	4.1
5	Marchand Pool	Green algal streamer	too small
6	Exit point of	Surface water (clear)	5.3
7	acid waters	Bottom water (clear)	5.5
8	from mine	Bottom mud (orange)	4.9
9	Junction of mine water and Marchand Pool	Bottom water (clear)	5.7
10	Marchand Pool	Top scum (oily)	4.6
11	Marchand Pool	Surface water (orange)	5.7
12	Marchand Pool	Bottom mud (orange)	5.8
13	Marchand Pool	Bottom water (green-brown)	4.9
14	Marchand Pool	Bottom water (green)	5.0
15	Pool spillway to Sewickley Creek	Bottom water (green)	5.0

After collection, the samples, were examined microscopically. The presence of viable bacteria was noted in samples 1, 2, 4, and 12. Sample 5 contained not only bacteria but green streamers recognizable as algae; numerous protozoa and planarians were present. The samples were used to inoculate duplicate tubes of culture media.

The various media were incubated at room temperature with the exception of 4 mm. OD pyrex tubes of agar medium SS-1, which were incubated at 30°C. Tubes of medium 9KS° were incubated on the shaker. The subcultures to the various media were observed daily and examined microscopically as required.

Observations made on the various media after one week of incubation at room temperature are summarized in Table III. Microscopic observations were made on the pyrite and iron media (9KP and 9K), although in some cases, simple visual examination was sufficient to recognize the deep red-orange precipitate formed by iron oxidation.

Table III. Growth in various culture media one week[a] after inoculation with acid mine water samples

	Growth in media containing the energy sources designated						
	$S^{=}$	Pyrite	Pyrite	Fe^{++}	$SO_4^{=}$ (anaerobic tubes)	$SO_4^{=}$ (capillary tubes)	S
Sample							
1	-	+	+	+	-	-	+
2	-			+	+	+	+
3	-			+			+
4	-	+	+	+	+	+	+
5	+			+	-	-	+
6	-	-	-	-			+
7	-	+	+	+			+
8	+	+	+	+	+	+	+
9	-	-	-	-			-
10	-			+			-
11	-			+			+
12	+	+	+	+	+	+	+
13	-	-	-	-	-	-	-
14	-			-	-	-	+
15	-	-	-	-			+

a Medium 9KS° examined after 10 days of shaking.

The cultures were carried through serial transfer on the appropriate media; determination of growth was made by a combination of microscopic examination and visual inspection.

The activity of resting cells of Ferrobacillus ferrooxidans on pyrites was tested by means of the Warburg technique. This technique is excellent for the determination of: (1) the ability of selected cultures to oxidize pyrite, (2) differences in the susceptibility of the various pyrites to oxidation, and (3) the physiology of pyrite oxidation by bacteria in short-term experiments. Pyrite samples (65 mesh) were weighed and placed in the Warburg vessel with sufficient cell suspension (pH 3.5) and water (pH 3.5) to bring the volume to a predetermined value. Cell suspensions for the experiments were adjusted to 0.4 mg. bacterial-N/ml. Oxygen uptake was measured in the conventional manner. Results of two such experiments with concentration of pyrites and cells as variables are shown in Figures 1 and 2. The rate of pyrite oxidation was increased only two-fold by a twenty-fold increase in pyrite concentration with these 65 mesh samples.

A series of Warburg experiments demonstrated the role of particle size of the substrate on oxidation rates. The results (shown in Table IV) demonstrate that reduction in particle size enhanced bacterial action six-fold in the case of the pyrite concretion and 35- to 70-fold when the pyrite concentrates were used as substrates. Oxidation with the bacteria was 20 to 40 times that of the controls for 3 of the 4 pyrites tested.

Table IV. Oxidation of pyritic material of two particle sizes in the presence of Ferrobacillus ferrooxidans

Sample No.	Microliters oxygen taken up in three hours[a]			
	Approximately 65 mesh		Passing through 325 mesh	
	No cells	Cells	No cells	Cells
29	28+	40	60	1342
30	12	16	51+	1137
34	23+	11+	91	29+
35	24+	336	46	2040

a Oxygen uptake/1.2 mg. bacterial-nitrogen/three hours.
+ Apparent evolution of gas.

In separate shaken-flask experiments the release of hydrochloric acid-soluble iron was determined instead of oxygen uptake, as a means of demonstrating oxidation of the pyrites. Results of iron determinations in one such experiment are shown in the following table (Table V). The 25 ml. conical flask used contained 100 mg. of pyritic material, an aliquot of cell suspension containing 0.84 mg. bacterial nitrogen and sufficient H_2SO_4-acidified water, pH 3.5, to bring the total volume to 4.0 ml. After 24 hours, 4.0 ml. of 2.0N HCl was added and the mixture heated 30 minutes on a steam bath. The HCl-soluble iron content of suitable filtered aliquots was determined as given in the section on Methods.

TABLE V. Production of hydrochloric acid-soluble iron from pyritic material in the presence of Ferrobacillus ferrooxidans

Sample No.	Cells	Micrograms HCl-soluble iron released[a]			
		Initial	24 hours	Net	Pyrite oxidized, %
29	+	1024[b]	4400	3376	9.42
	-	808	1080	272	0.76
30	+	1250	4000	2750	9.84
	-	1034	1376	342	1.22
35	+	3040	7200	4160	11.99
	-	2824	3140	316	0.91

a Production of iron from 100 mg. pyritic material in the presence of 0.84 mg. bacterial-N.

b 216 /ug.. Fe were carried over with the cell suspension.

The ability of Thiobacillus thiooxidans to accelerate the oxidation of the pyrite samples was also tested in Warburg experiments. It was found that this organism was unable to accelerate oxidation rates over those of control vessels. The ability of T. thiooxidans and F. ferrooxidans to accelerate oxidation of museum grade samples (large crystals free of inclusions and contaminating materials) of pyrite and marcasite was tested. It was found that both strains attacked the marcasite, Ferrobacillus being more effective; neither culture accelerated the oxidation of museum grade pyrite. Results of these experiments are seen in Table VI.

Table VI. Effect of Thiobacillus thiooxidans or Ferrobacillus ferrooxidans on the oxidation of museum grade sulfide minerals

Sample	Cells	Microliters oxygen taken up in 3 hours per mg. bacterial-nitrogen	
		T. thiooxidans	F. ferrooxidans
Pyrite	-	15	38
	+	70[a]	19[a]
Marcasite	-	82	209
	+	128	734

a Apparent gas evolution.

Efficacy of use of a mixture of the two strains was also tested. In these Warburg experiments a resting cell suspension of Ferrobacillus was placed in the vessel and oxygen uptake was measured for one hour. At the end of this time the sulfur-oxidizing Thiobacillus was tipped into the reaction mixture. The effect on oxidation of samples 29, 30, 35, and museum grade marcasite was determined. The effect of addition of Thiobacillus is seen in Figure 3.

The rate of oxidation of ferrous iron and elemental sulfur by the strains of Ferrobacillus ferrooxidans and Thiobacillus thiooxidans in use in the experiments has been calculated from the data obtained in thepreceding experiments. These are presented in Table VII.

Table VII. Rates of oxidation of ferrous iron and elemental sulfur by Ferrobacillus ferrooxidans and Thiobacillus thiooxidans

Organism	$Q_{O_2}(N)^a$ S (1000 μmoles)	$Q_{O_2}(N)^a$ Fe^{++} (500 μmoles)
T. thiooxidans	557	0
F. ferrooxidans	148	3672

a Q_{O_2} (N) represents μliters oxygen uptake/mg. cell-nitrogen/hour.

Other manometric experiments with polycyclic aromatic hydrocarbon-grown cells of a strain of Pseudomonas aeruginosa were carried out to study the activity of bacteria regarding sulfur-containing organic compounds. It was found that cells grown with phenanthrene as source of carbon would take up more oxygen than cells grown on naphthalene when benzthiophene was provided them as a substrate. Benzthiophene was chosen as a model sulfur containing aromatic of the type thought to be present in coal. If oxygen uptake of both crops with naphthalene as substrate is calculated to a value of 1.0, then uptakes on benzthiophene were: naphthalene-grown, 0.3; phenanthrene-grown, 1.3.

Benzthiophene was provided at a concentration of 3.3 μmoles/ml. At 6.6 μmoles/milliliter, less oxygen was taken up than at the lower concentration. At 3.3 μmoles/ml. addition of benzthiophene to the flask contents served to lower oxygen uptake of cells metabolizing naphthalene and 2-methylnaphthalene (Figure 4). Thus it seems that a competitive inhibition or perhaps simple toxicity of the compound may be evident except in the case of cells grown on or metabolizing phenanthrene (Figure 5).

DISCUSSION OF RESULTS

Results obtained on examination of the microflora of acid mine drainage showed that top waters and rapidly running waters (green) were almost sterile in all cases. The best sources of microorganisms are the muds, which are apparently rich in all forms tested for. The bottom muds (orange) collected at the pool accumulate only where the water is not turbulent or flowing too rapidly. Where water flow is rapid, bottom mud does not accumulate, the orange color of oxidized iron products is absent, and the number of microorganisms present is quite small. In the laboratory, samples of such moving clear water after two weeks of incubation give evidence of iron oxidation (orange precipitate, heavy). The reason for their clarity is probably that they are simply flowing too rapidly for oxidation of iron to occur. Thus, clarity of water is no criterion of its potential yield of ferrugineous mud under non-agitated conditions.

The finding that all types of bacteria tested for appear to be present in the samples illustrates that those chemicals present in an environment are usually accompanied by bacteria capable of utilizing them. The importance of these particular types found, in regard to the problems of sulfur removal from coal and treatment of acid mine waters, is as follows:

(1) The same organisms which play a role in the production of acid mine waters, and in whose presence oxidation of large sulfuritic concretions, pyrite, and marcasite has been demonstrated are precisely those forms whose abilities must be exploited in any attempt to desulfurize coal by microbiological means.

(2) In any plan for disposal of sulfuric acid microbiologically, the use of anaerobic sulfate-reducing bacteria to convert sulfuric acid to elemental sulfur and/or hydrogen sulfide must be considered. The isolation of such bacteria from the bottom mud samples collected gives hope that strains of these organisms able to tolerate the acid environment necessitated by the use intended can be found or developed. Acid conditions are known not to be optimum for their growth, but their presence in samples 4 and 5 at pHs of 4.1 and 4.9, respectively, indicate they may be more tolerant of acid than previously assumed.

(3) The presence of sulfur oxidizers, iron oxidizers, and sulfate reducers in a given environment (ferrugineous mud) indicates that all three forms may participate in a cyclical process for sulfur. Thus, it appears that previous testing of pyrite oxidation with pure cultures of bacteria may not provide the best means possible for carrying out the process. It may well be better to try the natural flora for most efficient activity, although mixed cells of Ferrobacillus and Thiobacillus did not have the desired affect on pyrite (Figure 3).

The manometric studies (Figures 1 and 2) with 65 mesh sample #35 demonstrated that oxygen uptake is significantly greater than that required solely for the oxidation of ferrous iron initially present in the pyrite sample as determined by our extraction procedure.

Significantly higher oxidation rates were obtained when the sulfuritic materials were ground to pass 325 mesh (Table IV). The ability of Ferrobacillus to accelerate the oxidation of the pyrite in samples 29 and 30 is encouraging as regards microbial coal desulfurization. These samples are pyrite concentrates from coal. These contain minute crystals of pyrite embedded in the coal with perhaps only one face exposed for oxidation. Yet in the presence of the bacteria oxidation proceeds at a rapid rate as compared to the rates in the absence of the organisms.

Based on the results found in these studies (Tables III-VII, Figures 1-5) certain generalizations regarding microbial processes for coal desulfurization may be drawn. These are that: 1. The rate of pyrite oxidation depends upon available, that is, exposed pyrite. As a corollary to this, the coal must be in a finely divided state in order to expose a maximum of the embedded pyrite. 2. The rate of oxidation of pyrite depends upon the "type" of pyrite present. The word type is used for want of a better description, since X-ray diffraction patterns are identical for the pyrite of samples 29, 30, 34, 35, and museum-grade pyrite.

Only sample #34 and museum-grade pyrite resisted oxidation in the presence of F. ferrooxidans. Sample #34 contained considerable calcite. Preliminary experiments after the calcite had been removed (neutralizing effect of $CaCO_3$) gave indication of oxidative activity.

The ubiquitous presence of Thiobacillus thiooxidans in acid-mine waters is as yet unexplained. Our results show that only marcasite, a substance rarely present in acid-producing areas, can be attacked by this organism, raising the question of a possible energy source for the organism. The production of free sulfur during the oxidation of pyrite is one explanation. However, the ability of the iron-oxidizing F. ferrooxidans to oxidize elemental sulfur suggests another explanation.

The three organisms found in acid mine waters are listed below together with their proposed energy sources.

Organisms	Energy source
Thiobacillus thiooxidans	Elemental S
Thiobacillus ferrooxidans	Ferrous iron; thiosulfate
Ferrobacillus ferrooxidans	Ferrous iron

The three are morphologically indistinguishable; the chief criteria for their description being energy source. During isolation procedures, it is entirely possible that F. ferrooxidans will grow in an iron medium and be identified as F. ferrooxidans and also be isolated in an elemental S medium and be identified as T. thiooxidans. Thus, F. ferrooxidans may really be a variant of T. thiooxidans and exists in acid-mine waters by virtue of its iron oxidizing capacity. The inability of Thiobacillus thiooxidans to oxidize iron may be due to the loss of adaptive iron-oxidizing enzymes upon growth in elemental sulfur.

The ability of bacteria to accelerate the oxidation of sulfuritic material has been studied primarily by investigators at the Mellon Institute (1) and West Virginia University (9). Their studies involved testing for the production of free iron, sulfate, or increased acidity from insoluble pyrite materials in the presence or absence of bacteria in experiments of long duration. The development of methods and media for the propagation in high yield of the relevant bacteria (5, 7) has enabled us to utilize the manometric method for examining the role of bacteria in the oxidation of pyrites, thus enabling us to accumulate comparable data in a short time. A comparison of the results obtained with those of the Mellon Institute and West Virginia University is given in Table VIII. In general, our findings agree most closely with those of the Mellon Institute.

Table VIII. Acceleration of the rate of oxidation of different pyritic materials by iron- and sulfur-oxidizing bacteria

	Ferrobacillus ferrooxidans			Thiobacillus ferrooxidans			Thiobacillus thiooxidans		
	M.I.	W.V.U.	B.M.	M.I.	W.V.U.	B.M.	M.I.	W.V.U.	B.M.
Sulfur ball	+	N.T.[a]	+	N.T.	+	N.T.	-	+	-
Waste material	N.T.	N.T.	+	N.T.	N.T.	N.T.	N.T.	N.T.	-
Museum-grade marcasite	+	N.T.	+	N.T.	N.T.	N.T.	+	+	+
Museum-grade pyrite	-	N.T.	-	N.T.	+	N.T.	-	N.T.	-

a N.T. = not tested.

ACKNOWLEDGMENTS

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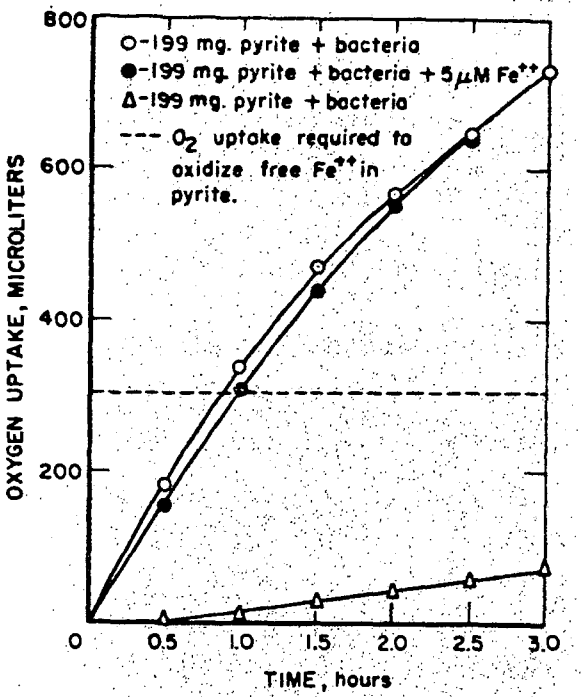

Figure 1.- Oxidation of sulfur ball (199 mg.) by Ferrobacillus ferrooxidans.

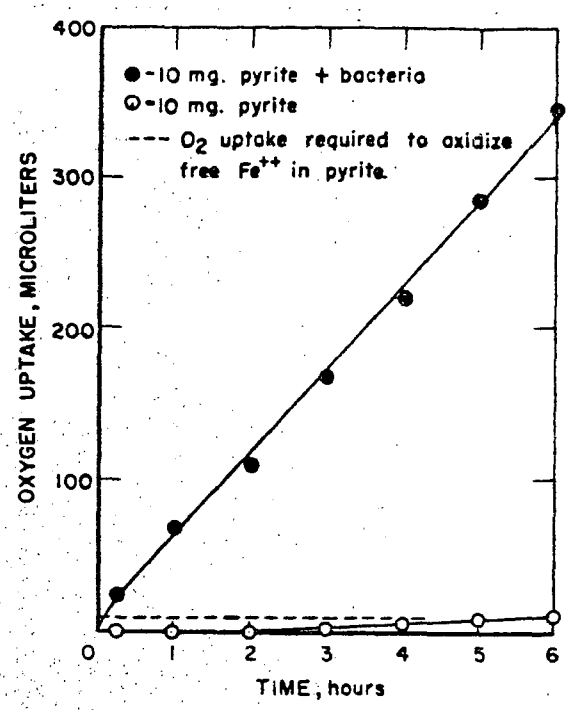

Figure 2.- Oxidation of sulfur ball (10 mg.) by Ferrobacillus ferrooxidans.

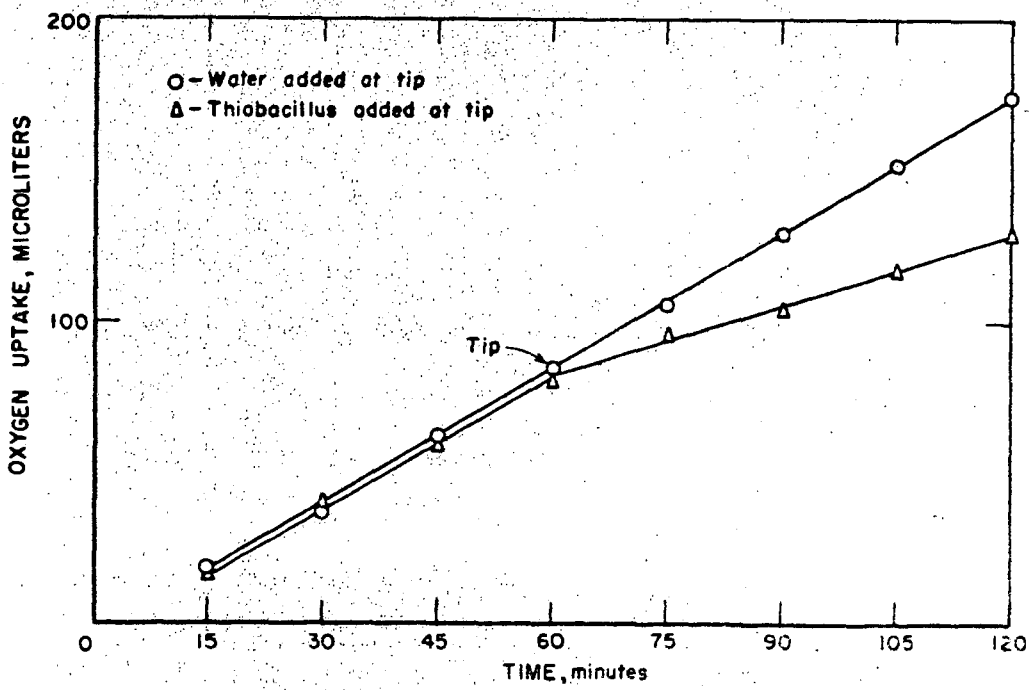

Figure 3.- Oxidation of sulfur ball (20mg.) by Ferrobacillus ferrooxidans.

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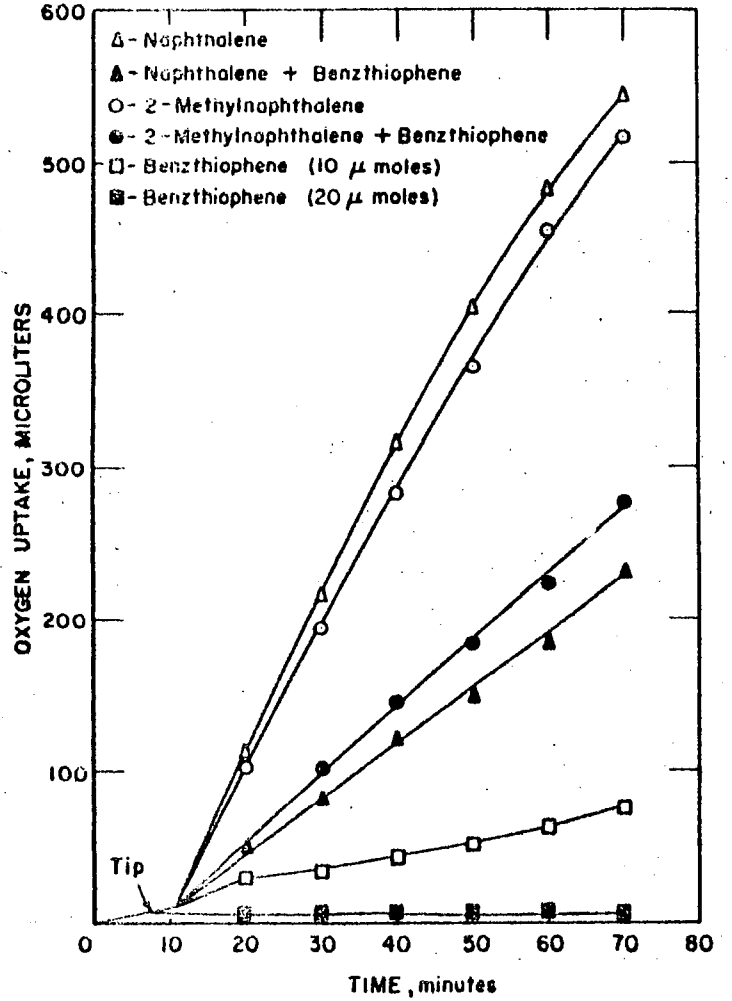

Figure 4.- Oxidation of aromatic hydrocarbons by strain PM-1.

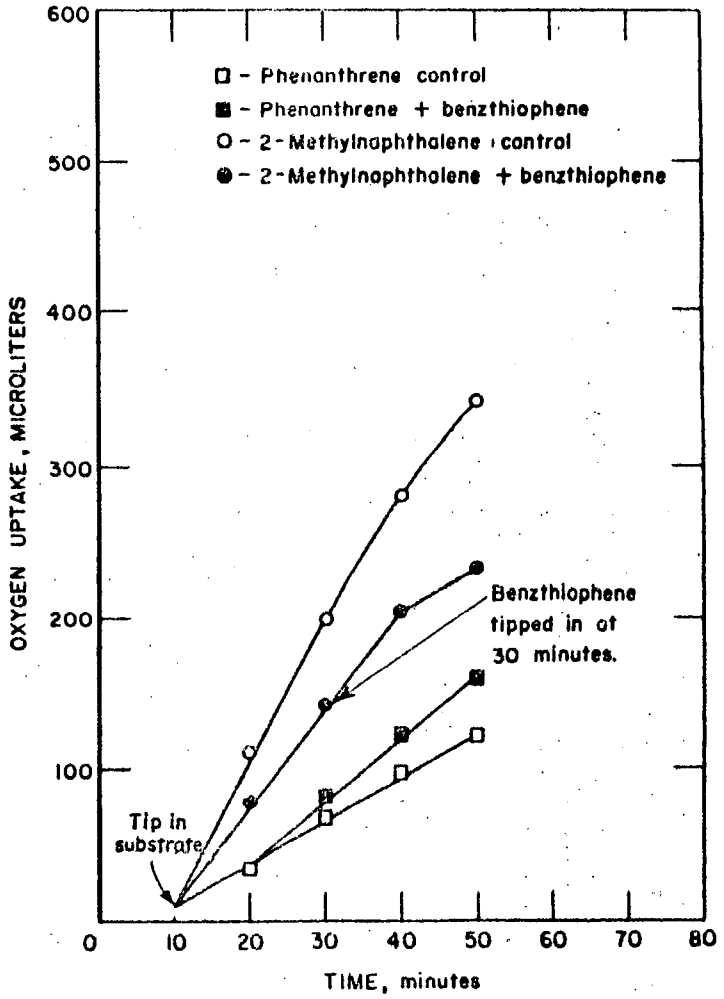

Figure 5.- Oxidation of aromatic hydrocarbons by strain PM-1.

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